Notch signaling activated by replication stress-induced expression of Midkine drives Epithelial-Mesenchymal Transition and Chemoresistance in Pancreatic Cancer

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Running title: Midkine expression is linked to EMT and chemoresistance.

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Abstract

The incidence of pancreatic ductal adenocarcinoma (PDAC) nearly equals its mortality rate, partly because most PDACs are intrinsically chemoresistant and thus largely untreatable. It was found recently that chemoresistant PDAC cells overexpress the Notch-2 receptor and have undergone epithelial-mesenchymal transition (EMT). In this study, we show that these two phenotypes are interrelated by expression of Midkine, a heparin binding growth factor that is widely overexpressed in chemoresistant PDAC. Gemcitabine, the front line chemotherapy used in PDAC treatment, induced Midkine expression in a dose-dependent manner and its RNAi-mediated depletion was associated with sensitization to gemcitabine treatment. We identified an interaction between the Notch-2 receptor and Midkine in PDAC cells. Midkine-Notch-2 interaction activated Notch signaling, induced EMT, upregulated NF-κB and increased chemoresistance. Taken together, our findings define an important pathway of chemoresistance in PDAC and suggest novel strategies for its clinical attack.
Introduction

Insufficient diagnostic tools and therapeutic options for pancreatic ductal adenocarcinoma (PDAC) still substantiate its ranking as 4th leading cause of cancer related death (1). The combination of an unusual aggressiveness and early metastatic locoregional as well as distant spread reflects the urgent necessity of new therapeutic options for this deadly disease since its incidence nearly equals mortality. This devastating prognosis is partially due to a frequent occurrence of intrinsic or acquired chemoresistance in PDAC specimens against the nucleoside analogue gemcitabine which is still the standard for chemotherapeutic treatment of locally advanced and metastatic PDAC (2).

The heparin binding growth-factor Midkine (MK) was first identified as a retinoic-acid inducible gene during embryogenesis two decades ago (3). Since then a plethora of cellular MK functions have been described in normal and transformed tissue. While MK gene-expression is weak or undetectable in adults, the MK re-expression in damaged and inflamed tissues have been discussed (4-5). In several malignancies including neuroblastomas, colorectal and breast carcinomas, protumorigenic effects have been linked to MK re-expression although the mechanisms are not completely understood (6-8).

During embryonal development the epithelial-mesenchymal transition (EMT) is an indispensable mechanism in which extracellular signals convert epithelial into mesenchymal cells. In adult organisms EMT take part in the pathology of certain diseases (9). During pathological processes such as cancer, EMT has been shown to orchestrate a process by which epithelial cells undergo morphologic changes: i.e. a transition from an organized cell layer and polarized organization of the cytoskeleton, which is linked to expression of the epithelial adhesion molecule E-cadherin, β-Catenin and γ-Catenin, to mesenchymal cells without an organized cell layer, showing enhanced pseudopodia formation and subsequently more motility and invasiveness which is linked to loss of epithelial cell-cell junction, reorganization of cytoskeleton and upregulation of mesenchymal markers Vimentin,
Fibronectin and α-smooth-muscle-actin (α-SMA) (10-12). EMT is triggered by a tightly regulated interplay of extracellular signals such as soluble growth-factors like epidermal growth-factor or transforming growth-factor β (13-14). Numerous receptors such as Notch have been identified playing a significant role in the acquisition of EMT in cancer (15). The activation of Notch regulates expression of target genes playing important roles in embryonic development, cell proliferation and apoptosis. Recently, the activated Notch receptor-2 (Notch-2) was shown to mediate an EMT phenotype in PDAC cells whereas chemotherapy (oxaliplatin)-induced Notch-1 activation was shown to be linked to acquired chemoresistance in colon cancer cells (16). Moreover, Notch-2 was recently shown to interact with MK to regulate cell plasticity and motility in human keratinocytes (17).
Materials and Methods

Cell lines. Tumor-tissues for establishment of primary chemoresistant PDAC cell lines (PaCa 5061, 5072, 5156) were taken from patients who underwent total pancreaticoduodenectomy for advanced PDAC in the Surgery, University Medical Center Hamburg (2009). Written informed consent of patients was obtained prior to surgery. The procedure of cell line establishment from tumor-tissue was previously published (18). Primary cells were cultured in TUM medium. L3.6pl (19), BxPC-3 and PANC-1 cells were cultured in DMEM or RPMI 1640 (Invitrogen) supplemented with 10% FCS and 200 IU/ml Pen-Strep at 37°C and 5% CO₂. For development of gemcitabine-resistant L3.6pl cells (L3.6pl-Res), cells were exposed to an initial gemcitabine-concentration of 0.05µM/L. The surviving population was grown to 80% confluence and passaged 3 times over 14 days to ensure stable viability. Cells were then treated with sequentially increased gemcitabine-concentrations (0.1µM/L, 0.3µM/L, 0.5µM/L, 1µM/L; 21 days each) up to the clinically relevant concentration of 2µM/L for 30 days. L3.6pl-Res cells are ~20 times more resistant to gemcitabine than L3.6pl cells. All used cell lines were genotyped by DNA fingerprinting (Identifiler™-Kit, Applied Biosystems).

Reagents. Gemcitabine was purchased from Eli Lilly and 5-FU from Sigma. Recombinant human Midkine (rh-MK) was purchased from R&D Systems and used at concentrations of 20 and 50ng/ml, respectively. In rh-MK experiments, cells were serum-deprived for 14h and treated for 24h with 20 or 50ng/ml.

Notch-2 signaling. PaCa 5061 and PANC-1 cells (10⁵) were cultured in 6-well plates. Then cells were cultured in serum-free media for ~14h. Subsequently, cells were treated for 24h with 20 and 50ng/ml of rh-MK or left untreated and served as control. rh-MK-induced Notch-2 cleavage was detected using antibodies recognizing the intracellular Notch-2ICD.
Functional receptor-activation was detected using antibodies against the downstream target-genes Hes-1 and NF-κB/RelA.

**RNA isolation and expression analyses.** Total RNA were isolated using TRIzol® (Invitrogen). The dried pellet was cleaned with the RNeasy MiniElute Kit (Qiagen). RNA-concentration was measured on a NanoDrop® Spectrophotometer (Peqlab). Gene-expression profiles of whole human-genome were generated by hybridizing either 5µg of total RNA from PaCa 5061, 5072 and 5156 or 5µg human total pancreas RNA (Stratagene and Ambion) serving as healthy controls on GeneChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix). Analyses were performed according to the One-cycle cDNA synthesis protocol for GeneChip® Expression-Analysis (Affymetrix) and achieved data were analyzed using GCOS 1.4.

**Cell viability analyses.** To determine drug sensitivity, cells were incubated with different concentrations of gemcitabine and 5-Fluorouracil (5-FU). Cell proliferation was determined with CellTiter 96® AQueous Cell-Proliferation-Assay according to the manufacturer’s protocol (Promega). Values for control cells were considered as 100% viability.

**Real-time reverse transcription-PCR (Real-time RT-PCR).** Real-time RT-PCR was conducted to quantify gene-expression or to verify RNAi-mediated downregulation. 1µg of total RNA was reverse-transcribed using the Transcriptor-cDNA-Kit (Roche). PCRs were carried out in a Mastercycler® ep-realplex (Eppendorf). Data were analyzed according to the comparative C_T method and normalized for Cyclophilin expression in each sample.

**Plasmids.** Cells were transfected with siRNAs against MK and Notch-2 or control siRNA (Santa Cruz) using Lipofectamine (Invitrogen). Notch-2 open-reading-frame encoding the N-terminal
extracellular domain (1-351) was amplified by PCR and subcloned into p3xFLAG-CMV-14. Midkine was amplified by PCR and subcloned for expression into pCS2 (20).

**Immunoprecipitation (IP) and Western blotting.** PANC-1 cells were transfected with indicated plasmids. Following transfection, cells were lysed in RIPA buffer (Sigma) containing 1x protease inhibitor cocktail (Roche). Supernatants were resolved by SDS-PAGE followed by immunoblotting or immunoprecipitation as previously described (20). Western blots, IPs and immunostainings were performed using antibodies listed in supplementary Table S2.

**Immunohistochemistry.** Stainings were performed with peroxidase method according to manufacturer’s protocol (R&D Systems). Paraffin-embedded human PDAC specimens were obtained from the Tissue-Bank of the University of Hamburg. PDAC slides (5µm) were deparaffinized with xylene and rehydrated with ethanol. Antigen-retrieval was done by microwave heating. Endogenous peroxidase activity was blocked by H2O2 [1% (v/v) in methanol]. Sections were counterstained with Meyer’s haemalaun-solution (Merck) and permanently mounted. For negative control stainings, normal tissue were stained or appropriate control IgG-stainings were performed.

**Data analyses.** Expression analyses and achieved data were analyzed using GCOS 1.4 and scaled to a default target signal-value of 150. Absolute and comparative analyses were performed using MAS 5.0 algorithm. Annotations were analyzed with interactive query analysis. Experiments presented are representative of three different repetitions. Data are presented as the mean values ± SE.
Results

Midkine is frequently overexpressed in chemoresistant PDAC. The PDAC cell line PaCa 5061 has recently been characterized in-depth in vitro and in vivo (18). To identify novel players involved in intrinsic or acquired chemoresistance in PDAC, we analyzed the gene-expression profile of three newly established chemoresistant cell lines (PaCa 5061, 5072, 5156) in comparison to RNA expression patterns obtained from two independent normal pancreas specimens. The native intrinsic gemcitabine-resistance was examined in all lines prior to the analysis. To identify chemoresistance-relevant molecules we searched for “hits” over-represented more than 5-fold in all resistant lines compared to normal pancreas. Among the top 5 hits we identified MK as overexpressed in cell lines (Fig. 1A, left). The P values and fold changes of selected genes are shown in Table 1 (Supplementary Table S1). To further validate our microarray results, we re-tested MK-overexpression and performed real-time RT-PCR to quantify MK mRNA in the investigated lines (Fig. 1A, right). We expanded these analyses and quantified MK mRNA-expression in more than 30 additional PDAC tissues by real-time RT-PCR (Fig. 1B). We observed a statistically significant mRNA-overexpression in more than 50% of the investigated samples (P<0.05). To verify that the MK mRNA-expression resulted in robust protein-expression, we performed immunostainings from same tissues or investigated MK protein-expression in cell lines and showed that mRNA-overexpression resulted in robust protein-expression. Consistent with previous observations where MK mRNA was shown to be overexpressed in PDAC (21), we further show that the mRNA-overexpression resulted in robust protein-expression (Fig. 1C). Moreover, steady-state MK protein-levels were upregulated in chemoresistant PDAC lines and almost undetectable in sensitive cells (Fig. 1D).

Gemcitabine-induced MK expression is dose-dependent. Because all investigated cell lines are highly gemcitabine-resistant, we hypothesized that the MK-overexpression may play a
role in chemoresistance. To test this, we treated chemosensitive and chemoresistant lines with gemcitabine. Interestingly, we found that MK protein-levels were increased in a dose-dependent manner in chemoresistant cells (Fig. 2A, top). In contrast, treatment of chemosensitive cells failed to increase MK protein-levels (Fig. 2A, bottom). Moreover, MK protein-levels were only barely detectable in control-treated as well as gemcitabine-treated chemosensitive cells. As it is known that the MK-signaling is post-translationally extinguished by proteasom-dependent degradation (22), we examined whether gemcitabine-induced MK upregulation occurs at the mRNA-level. Indeed, we found that MK mRNA-expression is dose-dependently induced by gemcitabine in chemoresistant PDAC cells (Fig. 2B, top). In contrast, MK mRNA-levels in gemcitabine-treated chemosensitive cells remained either slightly decreased or unchanged (Fig. 2B, bottom). Moreover, we investigated whether other chemotherapeutics used in PDAC treatment may also increase MK expression. As expected, treatment of chemoresistant cells with 5-Fluorouracil (5-FU) resulted in dose-dependent MK upregulation, whereas no induction was again observed in chemosensitive cells (Supplementary Fig. S1). Because MK is a secreted growth-factor involved in paracrine/autocrine regulation of growth and differentiation, we investigated whether the MK-secretion is increased in chemoresistant cells upon gemcitabine treatment. Indeed, we found robust and dose-dependently released MK protein-levels in gemcitabine-treated cells (Fig. 2C, top), whereas no increased MK-secretion was detectable in chemosensitive cells (Fig. 2C, bottom).

RNAi-mediated abrogation of MK induction by chemotherapy restores chemosensitivity. Because MK is induced at the mRNA and protein-level in gemcitabine-treated chemoresistant cells, we hypothesized that MK mediates intrinsic chemoresistance. First, we examined whether RNAi-mediated MK downregulation can restore chemosensitivity in PDAC cells. Transient transfections of PANC-1 and PaCa 5061 with two different MK
siRNAs decreased efficiently MK expression (Fig. 3A and B, right). We then analyzed the consequences of depleted MK during chemotherapy and performed cell viability assays. Strikingly, we observed that MK-depletion can effectively restore chemosensitivity in chemoresistant cells (Fig. 3A and B, left). Moreover, we performed rescue-experiments via exogenous treatment of MK-depleted PaCa 5061 and PANC-1 cells with rh-MK. Here we observed substantially increased chemoresistance in rh-MK-treated MK-depleted cells, compared to untreated cells and chemoresistance reached almost similar levels as control-transfected cells (Supplementary Fig. S2). As we found the MK-secretion to be induced by gemcitabine, we examined whether BxPC-3 and L3.6pl cells may acquire a chemoresistance phenotype through exogenous treatment of rh-MK. Serum-deprived cells were pre-treated with rh-MK followed by treatment with increasing gemcitabine-concentrations and then transferred to viability assays. Interestingly, pre-treatment of L3.6pl cells with rh-MK resulted in substantially increased viability compared to untreated cells (Fig. 3C, left) whereas chemosensitivity of BxPC-3 cells was unaltered (Fig. 3C, right).

**MK-overexpression contributes to EMT in pancreatic cancer.** To identify molecular alterations which mediate chemoresistance and to investigate whether the MK expression is involved in this mechanism, we obtained a chemoresistant subclone from chemosensitive L3.6pl cells, hereafter designated as L3.6pl-Res, through sequential treatment with increasing gemcitabine-concentrations over time. In viability assays L3.6pl-Res cells showed a substantial enhancement of chemoresistance towards gemcitabine. However, during the establishment of L3.6pl-Res cells we observed morphologic changes towards spindle-shaped morphology and enhanced pseudopodia formation (Fig. 4A, right), whereas L3.6pl cells displayed a cobblestone epithelial morphology (Fig. 4A, left). As previously reported (23) the observed phenotypic changes suggested that L3.6pl-Res cells had undergone EMT over time. To further confirm these observations we analyzed in more detail by western blotting and
real-time RT-PCR, whether the acquisition of gemcitabine-resistance induced molecular changes consistent with EMT. As a hallmark of epithelial cells, we found elevated expression of the epithelial adhesion molecule E-cadherin in L3.6pl cells. In addition, β-Catenin and γ-Catenin protein-levels were prominently expressed, suggesting L3.6pl cells display an epithelial phenotype. In contrast, analysis of L3.6pl-Res cells revealed increased expression of mesenchymal markers Vimentin, Fibronectin and α-SMA (Fig. 4B). Moreover, expression of the EMT-related transcription-factors Snail and NF-κB were markedly increased. As Notch-2 is upregulated in gemcitabine-resistant cells (15), we also analyzed its expression and found substantially increased Notch-2 protein-levels in L3.6pl-Res cells, suggesting that its expression is not only important for gemcitabine-induced EMT but also for acquired chemoresistance in PDAC. Analysis of L3.6pl-Res cells for other Notch family-members did not reveal any and found no increased expression except for Notch-2 (Supplementary Fig. S3). Interestingly, gemcitabine-treatment of chemosensitive BxPC-3 cells failed to induce Notch-2 expression (Supplementary Fig. S4). We then investigated whether MK is re-expressed in L3.6pl-Res cells and found tremendous MK mRNA as well as protein-levels compared to L3.6pl cells (Fig. 4B and C). To pinpoint whether these molecular changes were due to changes in gene-expression, we quantified the expression of E-cadherin, Vimentin, Notch-2 and MK using real-time RT-PCR (Fig. 4C). E-cadherin was greatly decreased whereas Vimentin, MK and Notch-2 expression was highly increased in L3.6pl-Res cells. Interestingly, these results were independently confirmed by the expression data obtained for three chemoresistant PDAC cell lines (data not shown).

In proof-of-concept experiments, we depleted MK expression in L3.6pl-Res cells using two different siRNAs and treated cells with increasing gemcitabine-concentrations followed by viability assays. MK-depleted L3.6pl-Res cells showed substantially decreased chemoresistance and reached almost similar levels of chemosensitivity as L3.6pl cells whereas si-control transfections had no influence on chemoresistance (Fig. 4D). We also
performed rescue-experiments and treated MK-depleted L3.6pl-Res cells with exogenous rh-MK. As expected, chemoresistance is substantially increased in rh-MK-treated MK-depleted cells, compared to untreated cells (Supplementary Fig. S2). Our results suggest that gemcitabine-induced EM-Transition accompanied by acquired chemoresistance is feasible in a subset of PDAC cells and involves the re-expression of MK and increased Notch-2 expression.

**MK-depletion induces reversal of EMT to MET in L3.6pl-Res cells.** As several growth-factors have been shown to trigger EMT we hypothesized that MK may play a role in the acquisition of EMT in PDAC. To confirm this we decreased MK expression in L3.6pl-Res cells with siRNA and investigated mRNA and protein-expression in respect of EMT markers. It was previously shown that depletion of Notch-2 in EMT acquired cells induced the reversal to MET (15). We therefore decreased Notch-2 expression in L3.6pl-Res cells using siRNA. As shown in Figure 5 the applied MK and Notch-2 siRNAs were highly effective which resulted in decreased protein-expression compared to si-control (Fig. 5A and B). Subsequently, we verified by real-time RT-PCR and western blotting whether EMT was reversed to MET in MK and/or Notch-2-depleted L3.6pl-Res cells. Indeed, E-cadherin mRNA-expression was significantly increased in either MK or Notch-2 siRNA-transfected L3.6pl-Res cells compared to si-control (Fig. 5C). To further confirm that si-MK and/or si-Notch-2 transfected cells are capable to induce MET, we analyzed Vimentin expression and found significantly decreased mRNA-levels (Fig. 5C). Along with the mRNA-expression, we found elevated E-cadherin and dramatically decreased Vimentin protein-expression in siRNA-transfected L3.6pl-Res cells, which is consistent with the reversed transition from EMT to MET (Fig. 5A). To validate whether Notch downstream-signaling is affected in Notch-2-depleted cells, we analyzed the protein-expression of NF-κB/RelA. Based on previously published observations (24), we found substantially decreased NF-κB protein-
expression in si-Notch-2 transfected cells suggesting that Notch downstream-signaling is affected (Fig. 5A). Interestingly, we found decreased NF-κB protein-expression in si-MK transfected L3.6pl-Res cells suggesting that MK cross-talk with Notch to positively regulate Notch downstream-signaling (Fig. 5A). It is well-known that an increased migration/invasion potential is a hallmark of mesenchymal cells. Therefore, we performed migration/invasion assay using MK-depleted PaCa 5061, 5072, 5156 and L3.6pl-Res cells or si-control transfected cells. As expected, we observed that MK-depleted chemoresistant PDAC cells migrated and invaded significantly lower than control-transfected cells in vitro (Supplementary Fig. S5).

**MK mediates chemoresistance through binding and activation of Notch-2.** Because MK and Notch-2 expression is dramatically increased in chemoresistant PDAC cells and both are linked to EMT, we hypothesized that MK may trigger Notch-signaling and subsequently the activation of anti-apoptotic pathways such as NF-κB-signaling to promote chemoresistance in PDAC. We first analyzed a possible interaction between MK and the extracellular domain of Notch-2 by subcloning coding region for MK into pCS2 (pCS2-MK) and extracellular domain (residues 1-351) of Notch-2 (Notch-2-exD\(^{1-351}\)) into p3xFLAG-CMV-14 resulting in expression of Flag-tagged Notch-2-exD\(^{1-351}\). The Flag-Notch-2-exD\(^{1-351}\) expression construct was transiently co-transfected along with pCS2-MK into PANC-1 cells and protein-complexes were precipitated with antibodies against MK followed by immunoblotting for detection of Flag-tagged Notch-2-exD\(^{1-351}\) and vice versa. Appropriate control transfections were carried out by co-transfections of pCS2-MK along with p3xFLAG-CMV-14. Strikingly, we found robust interaction of MK and Notch-2-exD\(^{1-351}\) in PANC-1 cells while MK failed to interact with Flag alone (Fig. 6A, *left*). Same experiment was done vice versa and revealed positive interaction between MK and Notch-2-exD\(^{1-351}\) (Fig. 6A, *right*). To investigate whether the MK-Notch-2 interaction is functional in respect of Notch-2 activation which is
followed by the γ-secretase-mediated cleavage of its intracellular domain (Notch-2ICD), we treated chemoresistant cells with rh-MK and analyzed cells for the presence of Notch-2ICD with specific antibodies. Treatment of chemoresistant cells with rh-MK resulted in an intensified cleavage of Notch-2ICD compared to untreated (control) cells, whereas full-length Notch-2 protein was slightly decreased (Fig. 6B). We found that rh-MK-mediated cleavage of Notch-2ICD was dose-independent and efficiently induced Notch-2 intracellular cleavage in the same manner. We then examined whether the rh-MK-mediated increase of Notch-2ICD could force up NF-κB protein-expression which is known to be the central regulator of anti-apoptotic pathways, and found elevated NF-κB protein-levels in Notch-2-activated cells. To further validate MK-mediated Notch-2 cleavage and activation, we analyzed protein-expression of the well known Notch-target in mammals, Hes-1, and found elevated protein-levels in rh-MK treated cells (Fig. 6B).

As it was recently shown that elevated Notch-1 expression is linked to increased resistance against oxaliplatin in colorectal cancer (16), we analyzed whether RNAi-mediated Notch-2-depletion would affect chemoresistance in PDAC. We transiently transfected PaCa 5061 and PANC-1 cells with either si-control or si-Notch-2 and treated cells with gemcitabine (5µM) or left them untreated (Mock) followed by viability assays. The combination of si-Notch-2 and gemcitabine decreased cell viability of both resistant lines compared to si-control transfected cells (Fig. 6C). (P<0.05).
Discussion

Despite improved diagnostic tools for PDAC, the often existing unspecific and indistinct symptoms delay diagnosis and render the majority of cases irresectable (25). Also completely resected patients develop recurrent disease and eventually require palliative treatment. The majority of patients receive little or no benefit from adjuvant therapies mainly because most of the cancer cells have shown to be either intrinsically chemoresistant or become chemoresistant during therapy.

In this study we addressed the evident need of identifying novel players and to further comprehend their molecular role for chemoresistance in PDAC. It was recently shown that MK-secretion has cytoprotective functions in hepatocytes against cellular damage when exposed to heavy-metals or when the MK protein was applied to the location of ischemia injury in the heart (26-27). The external-stimuli and intracellular-signaling that may promote MK expression are unknown. Several receptors have been proposed to turn activated upon MK binding in embryonic tissue as well as in cancer. MK was shown to bind low density lipoprotein receptor-related protein, α4β1-integrin and α6β1-integrin in embryonic neuronal tissue which mediated intracellular phosphorylation of Src family proteins (28-29). For promotion of migration and invasion of human head and neck cancer cells, it was proposed that MK binds to tetraspanin which then induces tyrosine phosphorylation of focal adhesion kinase followed by activation of the STAT1α pathway (30). In this study we have found a robust interaction of MK and Notch-2 in vitro. The treatment of Notch-2 positive PDAC cells with soluble MK resulted in Notch-2 activation and was linked to upregulation of Notch downstream-targets Hes-1 and NF-κB/RelA, suggesting that the MK-Notch-2 interaction positively regulates Notch downstream-signaling. Notch-2 and Notch-4 expression were recently described to be upregulated in chemoresistant PDAC which is linked to acquired EMT (15). Our data confirmed this in case of Notch-2 but not of Notch-4. Although all Notch receptors are more or less expressed in the investigated cells we observed a specific Notch-2
upregulation suggesting a sine qua non in triggering and maintaining the EMT phenotype which is accompanied by increased chemoresistance. In proof-of-concept studies we found substantially increased chemosensitivity in Notch-2-depleted cells suggesting that Notch-signaling not only contributed to proliferation and differentiation but also to the regulation of cell-death through cross-talking with NF-κB-signaling. Our observations are therefore in agreement with previous studies showing Notch-signaling to positively influence NF-κB-signaling through the transcriptional regulation of positive regulators of the pathway in T-cell leukemia (31). In this context it was shown that NF-κB is not only frequently constitutive active in PDAC but also represses E-cadherin expression in mammary epithelial cells and, thus, supporting EMT (32-33).

According to recent publications, the natural Notch ligand Jagged-1 is upregulated in chemoresistant PDAC (15) and chemoresistant colorectal cancer (16). Obviously, downregulation of Jagged-1 expression in colorectal cancer revealed no functional relevance for chemoresistance (16). Interestingly, we also observed that Jagged-1-depletion in PDAC cells had no impact on chemoresistance (data not shown) suggesting that binding or recruitment of other molecules are of pivotal importance for mediating acquired chemoresistance in PDAC.

It is well known that the phenotype of chemoresistant and EMT acquired PDAC cells is associated with changes in their morphology. Through serial gemcitabine exposure of L3.6pl cells we also observed morphological changes that were associated with decreased expression of epithelial markers and an increase of mesenchymal markers suggesting that L3.6pl cells were capable to induce an EMT phenotype over time. Moreover, we found re-expression of MK and increased Notch-2 expression in EMT acquired L3.6pl-Res cells. Attempts to establish a drug-resistant cell clone from BxPC-3 cells were unsuccessful. This phenomenon was also mirrored by the observation that exogenous treatment of L3.6pl cells with rh-MK resulted in substantially increased chemoresistance whereas chemosensitivity of BxPC-3 was
unaltered. Therefore, we hypothesize that cells lacking corresponding signaling-cascades or a specific set of molecules are not capable to undergo EMT accompanied by increased chemoresistance in PDAC. Interestingly, it was already shown that MK and Notch-2 are independently involved in EMT processes. During early organogenesis in mice the MK expression was shown to be associated with epithelial-mesenchymal interactions. The authors observed that the MK gene was highly expressed in mesenchyme whereas the protein was distributed to the surface of epithelial cells which expressed non-detectable mRNA-amounts during several stages of tooth-development in mice suggesting that MK may act by a paracrine mechanism (34). Strikingly, it was shown that Notch-2 expression is also regulated by epithelial-mesenchymal interactions in the developing mouse tooth and is re-expressed in adults under pathological conditions suggesting that Notch-2 is involved in cellular repair processes during vertebrate development (35).

Our data are also in full agreement with previous observations where MK was identified being overexpressed in chemoresistant gastric cancer cells (36). Factors that may potentiate transcription or re-expression of MK are still unknown. Further, one could speculate that increased MK-secretion may also trigger intercellular chemoresistance by activating Notch-signaling in surrounding neighbouring cells in order to activate defense-mechanisms against drug-toxicity. Probably our results may also explain previous observations in which the treatment of chemosensitive cells with conditioned cell-culture media from MK overexpressed cells was linked to increased Akt-phosphorylation and, thus, increased cell viability (37).

In conclusion, our study revealed the identification of a novel mediator of EMT and drug-resistance in PDAC cells. To further corroborate our findings in vivo, knowledge-transfer to mice models are of pivotal importance. It should furthermore be determined whether these findings are applicable to other cells capable of developing resistance towards other chemotherapeutics. Therefore targeting MK or blocking/reversing EMT prior to or during
chemotherapy may force chemoresistant cells to revert to sensitive cells and, thus, may eventually be of tremendous benefit for patients suffering from advanced chemoresistant cancers, as it is unfortunately still the reality for PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Figure legends

**Figure 1. MK is frequently overexpressed in chemoresistant PDAC.**

_A_, Differential MK gene-expression in primary chemoresistant PDAC cell lines compared to two independent normal pancreas probes (control 1+2) obtained by microarray analyses. _left_: Mk is significantly over-represented in chemoresistant PDAC lines more than 5-fold (relative units) compared to normal pancreas ($P<0.004$). _right_: Real-time RT-PCR was used to quantify MK mRNA-expression in chemoresistant lines compared to normal pancreas. **, $P<0.01$; *, $P<0.05$.

_B_, Real-time RT-PCR based quantification of MK mRNA-expression in PDAC patient samples (n=31) compared to normal pancreas (n=8). Results are expressed as fold over control. *, $P<0.05$.

_C_, IHC of MK in selected patient tumors (5355, 5449, 5570, 5691) vs. normal tissue. IgG control-stainings revealed specificity of MK detection.

_D_, Western blots showing steady-state MK expression in PDAC cell lines.

**Figure 2. Dose-dependent MK expression in gemcitabine-treated resistant PDAC cells.**

_A_, Chemoresistant and sensitive PDAC lines were treated with increasing gemcitabine-concentrations (0.25-10µM) or left untreated (C). MK protein-expression was assessed using specific antibodies.

_B_, Real-time RT-PCR was conducted to analyze whether increased MK protein-levels were linked to higher MK gene-expression in chemoresistant and sensitive lines. Cells were treated with increasing gemcitabine-concentrations or left untreated (C) followed by RNA extraction. ($P<0.05$).

_C_, MK-secretion is dose-dependently increased in gemcitabine-treated chemoresistant cells verified by immunoblotting of conditioned cell culture media.
**Figure 3.** RNAi-mediated abrogation of MK induction by chemotherapy restores chemosensitivity.

* A, PANC-1 cells were transiently transfected with two different MK siRNAs or non-specific si-control. Then cells were treated with increasing gemcitabine-concentrations for additional 24h and viability was measured by MTT assay. *left:* RNAi-mediated MK-depletion is linked to increased chemosensitivity compared to si-control. *right:* RNAi-mediated downregulation of MK protein-levels.  

*B*, Same experiment as in A was performed with PaCa 5061, another chemoresistant line.  

*C*, Serum-deprived chemosensitive cells were treated with rh-MK (50ng/ml) for 14h followed by treatment with increasing gemcitabine-concentrations for 24h and then cell viability was measured.

**Figure 4.** MK expression is linked to morphologic and molecular changes consistent with EMT.

* A, L3.6pl cells display cobblestone epithelial morphology (*left*), whereas L3.6pl-Res cells display spindle-shaped morphology with loss of polarity and enhanced pseudopodia formation (*right*).  

*B*, epithelial and mesenchymal markers in L3.6pl and L3.6pl-Res cells. Note re-expression of MK and increased Notch-2 expression in resistant cells.  

*C*, Real-time RT-PCR was used to quantify gene-expression of E-cadherin, Vimentin, Notch-2 and MK in L3.6pl and L3.6pl-Res cells. ***, *P*<0.01; *, *P*<0.05.  

*D*, L3.6pl-Res cells were transfected with two different MK siRNAs or si-control. Then cells were treated with increasing gemcitabine-concentrations for 24h and cell viability was measured. Untransfected L3.6pl and L3.6pl-Res cells were also subjected to MTT assay. *left:* RNAi-mediated MK-depletion is linked to substantially increased chemosensitivity in L3.6pl-Res cells compared to si-control. *right:* RNAi-mediated downregulation of MK protein-levels.
Figure 5. MK-depletion is linked to decreased NF-κB levels and to reversal of EMT to MET in L3.6pl-Res cells.

A, L3.6pl-Res cells were transiently transfected with siRNAs against MK and Notch-2 or with si-control for 48h. RNA and protein-lysates were extracted. RNAi-mediated depletion of either MK or Notch-2 protein-levels. Mk-depletion did not influence Notch-2 expression and vice versa. B, Real-time RT PCR was conducted to quantify MK and Notch-2 mRNA-expression in L3.6pl-Res cells transfected with siRNA or si-control. **, P<0.01; *, P<0.05.

C, RNAi-mediated depletion of either MK or Notch-2 in L3.6pl-Res cells resulted in increased E-cadherin mRNA and decreased Vimentin mRNA-expression quantified with real-time RT-PCR. **, P<0.01; *, P<0.05.

Figure 6. MK interacts with Notch-2 ligand-binding domain and mediates chemoresistance through activation of Notch-signaling.

A, Interaction of full-length MK and Notch-2 N-terminal domain (Flag-Notch-2-exD1-351) in co-transfected PANC-1 cells. Total cell extracts were immunoprecipitated with specific antibodies or non-specific IgG antibodies. left: Western blot of MK IPs (top) and 100% input control (bottom) using antibodies against MK. right: Western blot of Flag IPs (top) and 100% input control (bottom) using antibodies against Flag. Lane 1: pCS2-MK and p3xFLAG-CMV-14; Lane 2 and 3: pCS2-MK and Flag-Notch-2-exD1-351. Lane 3 served for non-specific IgG precipitations.

B, Exogenously applied rh-MK-mediated cleavage of NotchICD in chemoresistant cells. Serum-deprived cells were treated with rh-MK (20 and 50ng/ml). Western blots of cell-lysates using antibodies against Notch-2, Notch-2ICD, NF-κB and Hes-1.

C, RNAi-mediated Notch-2-depletion in chemoresistant cells is linked to increased chemosensitivity. Cells were transfected with si-control or Notch-2 siRNA or left untreated (Mock). Transfected cells were treated with gemcitabine (5µM) for 24h and cell viability was measured. **, P<0.01; *, P<0.05.
Güngör et al.: Figure 1

**A**

Bar graphs showing relative mRNA expression of Midkine in control and patient samples.

**B**

A bar graph representing relative expression of Midkine in different PDAC samples.

**C**

Histological images comparing normal and Midkine-expressing tissues.

**D**

Western blot images of Midkine and Actin expression in Gemcitabine-sensitive and resistant cell lines.
Güngör et al.: Figure 3

A

PANC-1

% of viable cells

0,00 0,1nM 1nM 10nM 100nM 1μM 10μM 50μM 100μM

Gemcitabine

B

PaCa 5061

% of viable cells

0,00 0,1nM 1nM 10nM 100nM 1μM 10μM 50μM 100μM

Gemcitabine

C

L3.6pl

% of viable cells

0,00 0,1nM 1nM 10nM 100nM 1μM

Gemcitabine

BxPC-3

% of viable cells

0,00 0,1nM 1nM 10nM 100nM 1μM 10μM

Gemcitabine
A

IP: anti-MK
WB: anti-Flag

1 2 3
Notch-2-exD1-381

Input
1 2 3
Midkine

B

PaCa 5081
Control
rh.Midkine

Notch-2
Notch-2ICD
NF-κB/RelA
Hes-1
Actin

PANC1
Control
rh.Midkine

Notch-2
Notch-2ICD
NF-κB/RelA
Hes-1
Actin

C

PaCa 5081

% of viable cells

Mock control Notch-2 siRNA

Media Gemcitabine

PANC-1

% of viable cells

Mock control Notch-2 siRNA

Media Gemcitabine
Notch signaling activated by replication stress-induced expression of Midkine drives Epithelial-Mesenchymal Transition and Chemoresistance in Pancreatic Cancer


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